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# GYNE AND DRONE PRODUCTION IN *BOMBUS ATRATUS* (HYMENOPTERA: APIDAE)

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## Abstract

For over a decade, our research group has studied the biology of the native bumblebee, *Bombus atratus*, to investigate the feasibility of using it to pollinate crops such as tomato, strawberry, blackberry and peppers. Traditionally, captive breeding has depended on the use of captured wild queens to initiate the colonies. The goal of the current work is to investigate conditions required to produce new queens and drones in captivity. In this study, 31 colonies were evaluated under either greenhouse or open field conditions over a 15 month period. A total of 1492 drones (D) and 737 gynes (G, i.e., virgin queens) were produced by all colonies, with 16 colonies producing both drones and gynes (D&G), 11 producing only drones (D) and 4 producing neither. Some of the D&G colonies had more than one sexual phase, but no colonies produced exclusively gynes. More drones and fewer gynes were produced per colony under greenhouse conditions with the highest number of drones produced by D&G colonies. The numbers of immature stages per cell declined in colonies as increasingly more resources were allocated to the production of gynes and the maintenance of increased nest temperature.

**Keywords:** bee breeding, bumblebees, sex allocation, sexual forms

## INTRODUCTION

Non-parasitic bumblebees in the genus *Bombus* are morphologically a very homogeneous group of social bees. There are 239 species worldwide, of which 42 are Neotropical with 12 occurring in Colombia (Williams, 1998; Abrahamovich & Díaz, 2002).

Field studies on rearing the native bumblebee in Colombia began in 1998, with *B. atratus* recognized early on as a very promising pollinator species because it can develop strong colonies in artificial chambers, thrives in grasslands and has a broad geographical distribution (Cruz, Almanza & Cure, 2007).

Our study describes the growth and development of *B. atratus* colonies from the time the

queen was captured in the field through the sexual phase culminating in the production of gynes (G) and drones (D). The goal was to provide basic data to enable successful production of gynes and drones for commercial production.

## Biology of *B. atratus*

In Colombia, *B. atratus* is common in the Eastern range of the Andes, being most abundant from 1800 to 2800 meters above sea level (masl) (Liévano, Ospina & Nates, 1994; Abrahamovich & Díaz, 2002; Cruz, Almanza & Cure, 2007). In Facatativá, Bogotá's Savannah, Colombia, the sexual forms (gynes and drones) appear from November to January and from June to August during periods of low rainfall (González, Mejía & Rasmussen, 2004). In contrast to other *Bombus*

species in northern latitudes, colonies of *B. atratus* survive from 1 to 10 years and may produce two cycles of sexual forms per year (Zucchi, 1973).

A typical *B. atratus* successful colony has the following four phases of development (modified from Duchateau & Velthuis, 1988): 1) the *initiation phase* includes the solitary period of colony formation by the founder queen to the emergence of all the workers from the first cell; 2) the *linear phase* of colony is characterized by a linear increase in the cumulative number of cells per day; 3) the *sexual phase* starts when the first gyne/drone egg are laid by the founder queen and lasts to the emergence of all gynes, and 4) the *post-sexual phase* is when colonies can be reactivated by a new queen or by a laying worker, or decay due to competition among the workers.

In nature, *B. atratus* builds nests in rodent burrows 4-5 cm below the soil surface. The colony expands vertically as new cells are built upon older ones. Cell volume average  $5.2 \pm 0.1 \text{ mm}^3$  and 4 to 15 eggs are laid horizontally in each cell (González, Mejía & Rasmussen, 2004). A unique feature of bumblebees such as *B. atratus* is that as the larvae mature, they are progressively separated into individual cells (Sladen, 1912; Michener, 1974, 2007). Larvae enclose themselves after 6 - 9 days and continue

to develop in cells that are increased in size to accommodate larval growth (Fig. 1). Furthermore, *B. atratus* is a *pocket maker* species that builds wax pockets linked to the cells of developing larvae and are provisioned with pollen as food for the larvae (Sladen, 1912). During the establishment phase of the colony, the pockets are built and provisioned by the queen, and later by workers. Depending on the number of eggs in the cell, one or two pockets are built per cell (González, Mejía & Rasmussen, 2004).

The mean developmental time from oviposition to adult worker emergence is  $29 \pm 5$  days, but this varies with temperature and food availability. The average developmental time of larvae is  $13.9 \pm 4.4$  days, and  $8.7 \pm 3.6$  days for pupae (Riaño et al., 2014). These times vary with nest temperature that may be regulated by the colony. In Facatativá, Cundinamarca, Colombia, González, Mejía & Rasmussen (2004) reported a mean temperature of  $27.2^\circ\text{C}$  in *B. atratus* nest that was  $11.5^\circ\text{C}$  higher than ambient.

*B. atratus* has a medium length glossae that enables it in grasslands to forage on the commonly available red clover (*Trifolium pratense*) and to pollinate various horticulture crops. In Colombia, *B. atratus* has been evaluated as a pollinator of such crops as uchuva, *Physalis peruviana* (Camelo et al., 2004); strawberries, *Fragaria x ananassa* (Perez, 2014); tomatoes,

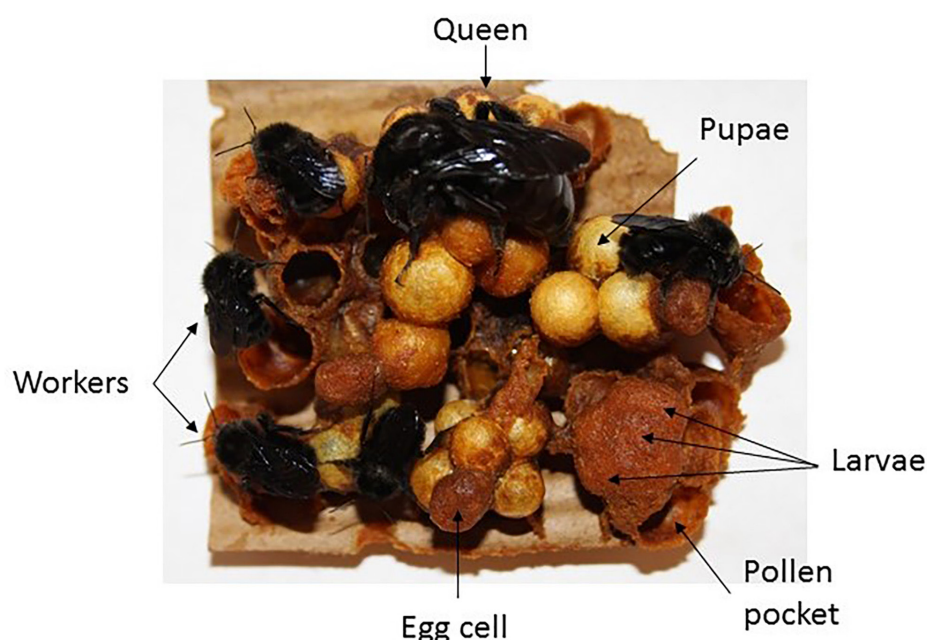


Fig.1 Colony structure of *Bombus atratus*

*Solanum lycopersicum* (Aldana et al., 2007); lulo, *Solanum quitoense* (Almanza, 2007); blackberries, *Rubus silvestris* (Zuluaga, 2011); red clover, *Trifolium pratense* (Lobatón, Cure & Almanza, 2012) and peppers, *Capsicum annuum* (Riaño et al., 2015).

## MATERIAL AND METHODS

### Study Area

The study was conducted during a 15 months period from April 2012 to July 2013 at the campus of the University of Nueva Granada located in the municipality of Cajicá (Cundinamarca, Colombia) at 2580 masl. The surrounding vegetation is mostly pastureland of the exotic tropical kikuyu grass (*Pennisetum clandestinum*) that is native to East Africa and interspersed with red clover. 21 colonies were placed in an open field where they were allowed to forage (Fig. 2, A and C), while another 10 colonies were housed in a 4000 m<sup>2</sup> greenhouse (Fig. 2, B) where they could forage on *Solanum quitoense*, *Brassica rapa*, *Raphanus sativus*, *Helianthus annuus*, *Dahlia imperialis*, *Phytolacca bogotensis* and *Borago officinalis* provisioned for this purpose (Romero et al., 2013). These

colonies also had access to the open field.

### Origin and maintenance of the bumble bee colonies

All colonies were initiated using queens collected in the pastureland around the university campus. It was not possible to know the age of the queens with certainty as some had not yet started a colony and others had not yet produced their first batch of workers and were in the process of initiating a colony. Newly captured queens were placed individually in 15x10x10 cm initiation boxes in a laboratory breeding room held at 26±2°C and 60±5% RH where they were fed commercially available pollen collected by from *Apis mellifera* hives and sucrose syrup (Pacateque et al., 2012). Each box was marked with where and when the queen was captured, as well as the dates of first oviposition and first worker emergence. When a colony had around five workers, it was moved to a larger 30x20x20 cm box where it was fed manually for 60 to 136 days. The differences in feeding time before moving a colony to whether the field or the greenhouse constitute a feeding time x foraging treatment.

Thirty-one colonies were moved to the field in



Fig. 2 Study areas at the Campus of the Nueva Granada University: 15 colonies were placed in open fields in zone A and 6 in zone C, with 10 colonies maintained in a greenhouse (B).



42x26x30 cm polystyrene boxes with a PVC tube that allowed the bumblebees access to the field. Nine colonies were moved to the field in September-October and twelve in December-February. The ten remaining colonies were placed in the greenhouse during December-February in 42x26x30 cm polystyrene boxes each having 2 PVC tubes that allowed workers to forage either in the greenhouse or in the field. The temperatures inside the boxes of some colonies in the field were monitored using a HOBO® data logger U12-012 (Onset Computer Corporation, 2013).

### Sampling data and statistical analysis

#### Sampling

Photographic records of all colonies were used to determine the timing of the important phenological events in each colony using the number of days after the capture of the founding queen (DAC) as a reference. Counts of immature stages, the dates of queen death and the emergence of the first gynes and drones were assessed twice a week, and the cumulative number of cells, larvae and pupae (workers + drones and gynes) and adults were plotted chronologically. Ignoring the stage subscript, the weekly data were used to estimate the changes in the numbers in each stage ( $\Delta S_t$ ) over time (eqn. 1a), with the cumulative numbers ( $S_t$ ) at time  $t$  computed using eqn. 1b (cf. Gutierrez et al., 1981).

$$\Delta S_t = \frac{(N_t + N_{t-\Delta t})}{2} * \Delta t / DEL \quad (1a)$$

$$S_t = S_{t-\Delta t} + \Delta S_t \quad (1b)$$

$N_t$  is the number of immatures in the stage at time  $t$  (DAC),  $N_{(t-\Delta t)}$  is the number at time  $t-\Delta t$  and  $DEL$  is the development time of each stage in days: egg = 7, larvae = 15, pupae = 10, gyne larvae = 17, gyne pupae = 16.

Gynes and drones in each colony were counted and removed twice weekly. The colonies were classified by location (field or greenhouse), and class as gyne and drone producing colonies (*D&G*), drone producing colonies (*D*) and colonies that produced only workers (*W*).

#### Statistical analyses

Nested ANOVA was used to evaluate wing length, head width, weight and the number of drones as a function of colony location and class. Six variables were analyzed to determine their importance in the *switching* of the colonies from worker to gyne production: (1) days in captivity, (2) initial number of workers ( $w$ ) produced during the period of captivity, (3) days to the emergence of the first worker, (4) days to emergence of the first drone, (5) longevity of the founder queen and (6) foraging location of the colonies. A Logistic Generalized Linear regression model (Equation 2) with backward elimination was used to determine the significance of the independent variables ( $x_1, x_2, x_3, \dots, x_n$ ) in predicting the probability of producing *D&G* colonies ( $P_{D\&G}(x)$ ) where  $a, b, c, d \dots h$  were fitted parameters (see; MacCullagh & Nelder, 1989).

$$P_{D\&G \text{ colonies}} = \frac{e^{a+bx_1+cx_2+dx_3+\dots+hx_n}}{1+e^{a+bx_1+cx_2+dx_3+\dots+hx_n}} \quad (2)$$

In addition, how the season (i.e., month) when the colonies were placed in the field affected the probability of gynes being produced and the number of gynes produced was analyzed using linear mixed models with Bayesian parameter estimation (Markov-Monte-Carlo chains with binomial or Poisson probability distributions; Hadfield, 2010).

#### Resource allocation

During colony development, more resources begin to be allocated to the rearing of gynes and less to the rearing of workers. To better describe this shift in resource allocation, we used as references the time of queen death ( $t_0$ ) and two additional reference times: 20 days before queen death ( $t_{-20}$ ) and 40 days before queen death ( $t_{-40}$ ). Using the data from the eight *D&G* and eleven *D* colonies at times  $t_0, t_{-20}$  the cumulative number of pupae (worker+drones) displaced +20 days ( $Y_T$ , where  $T = T_{+20 \text{ days}}$ ) on the numbers of cumulative cells ( $X_T$ , where  $T = t_0$  or  $t_{-20}$ ) were calculated. A covariance analysis using a linear mixed model for repeated meas-

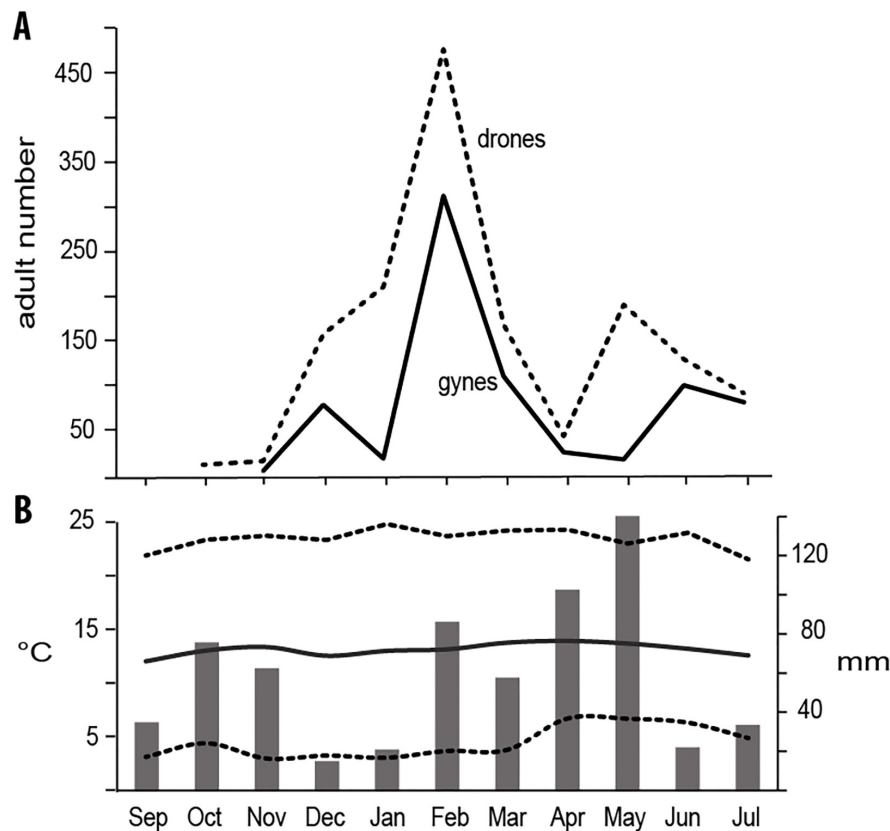


Fig.3 A: Total number of drones and gynes produced by all colonies between September, 2012 and July, 2013. B: Local weather during the period with bars representing mean rainfall, and lines represent temperatures: maximum, minimum and average.

measurements was done to compare the curves at different moments from queen death (Everitt, 2005). A multiple regression analysis was used to assess the shift in resource allocation in the eight *D&G* colonies for  $T = t_0$ ,  $t_{-20}$  and  $t_{-40}$ .

#### Temperature variation inside the colonies

ANOVA and Tukey tests were used to compare minimum, maximum and average temperatures inside the nest to ambient temperatures in six colonies with different initial sizes (two small, two medium and two large located in the open field) during the linear phase. The size of the colony was classified according to the number of workers when it was moved to the field. Temperatures in a *D&G* colony were compared to ambient during all phases of colony development.

## RESULTS

### Sexual forms

The total number of sexual forms produced by the 31 colonies during the fifteen months

study period was 1492 drones and 737 gynes. Sixteen colonies produced drones and gynes (*D&G*), eleven colonies produced only drones (*D*) and four colonies produced only workers (*W*). No colonies produced only gynes. Location did not affect the forming *D&G* colonies. One of the *D&G* colonies had a second cycle of drones and gynes, and another completed a third cycle, both of them located in the open field. Three *D* colonies were reactivated by a new queen and produced drones and gynes.

Gynes and drones were produced continuously throughout the year but were more abundant in December-March and May-July (Fig. 3). More *D&G* colonies were produced when the colonies were moved from captivity to the field in October ( $P < 0.01$ ) and December ( $P < 0.05$ ) (Fig. 4). Furthermore, more drones were produced per colony in the greenhouse than in the open field in both *D&G* and *D* colonies ( $P < 0.001$ ), but no significant difference was observed in the number of gynes in the two locations ( $P > 0.05$ ) (Fig. 4). There was no difference in size of gynes in the open field and greenhouse treatments ( $P > 0.05$ ).

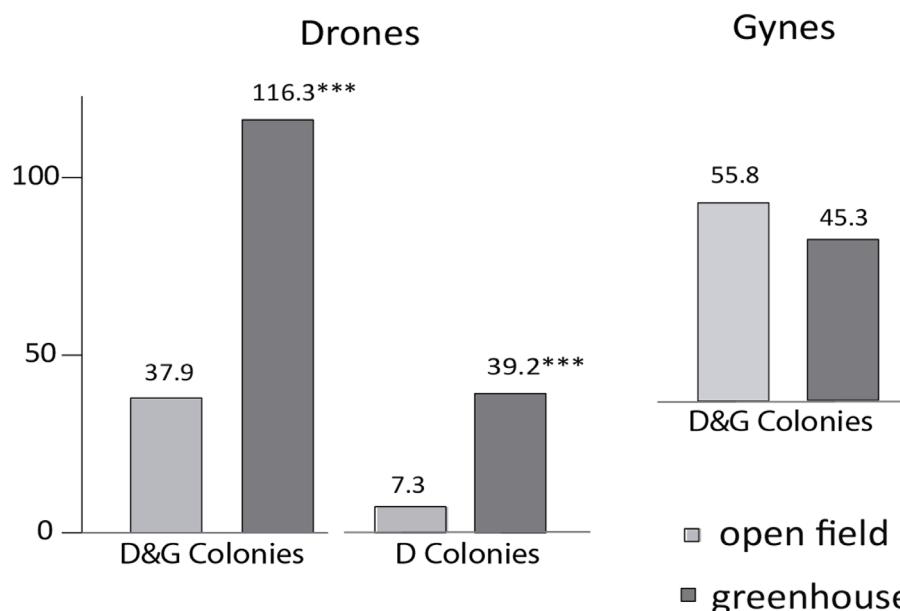


Fig. 4 Average number of drones and gynes produced per cycle per type of colony for D&G and D colonies (all months included), where \*\*\* indicates significant differences ( $P < 0.001$ ).

On the other hand, Drones were larger in *D&G* colonies than in *D* colonies (Wing length  $P < 0.05$ ; weight  $P < 0.05$ ) (Fig. 5).

Cells with larvae destined to become gynes have an operculated opening constructed for workers and used for direct feeding of the larvae (Fig. 6A). The operculated stage lasts for about 6.3 days and is followed by a prepupae-pupae stage of 16 days. The egg to adult cycle for gynes is about 40 days, approximately eight days longer

than for workers. Pots filled with pollen were constructed at the same time as the first operculated cells appeared (Fig. 6 D).

Table 1 summarizes the average characteristics of the different classes of colonies. The longevity of founder queens was similar in D&G and D colonies ( $P > 0.05$ ). However in all D&G colonies, the founder queens died before the emergence of the first gyne indicating that developing immature gynes did not require the presence of the queen.

On average, the first drones emerged 12 days after the first operculated cells were formed and twelve days before the first adult gyne emerged.

Of the six factors evaluated for their effect on the production of *D&G* colonies, only days in captivity and number of workers produced in the colony during the captivity period ( $w$ ) were significant. Since both these factors are highly correlated, only the number of workers was used in the logistic regression; specifically, the probability of producing *D&G* colonies decreased as  $w$  (Equation 3) increased (Fig. 7). Furthermore, colonies that were transferred early to the field produced more *D&G* colonies despite having fewer workers than colonies kept longer in the rearing chambers ( $P < 0.05$ ).

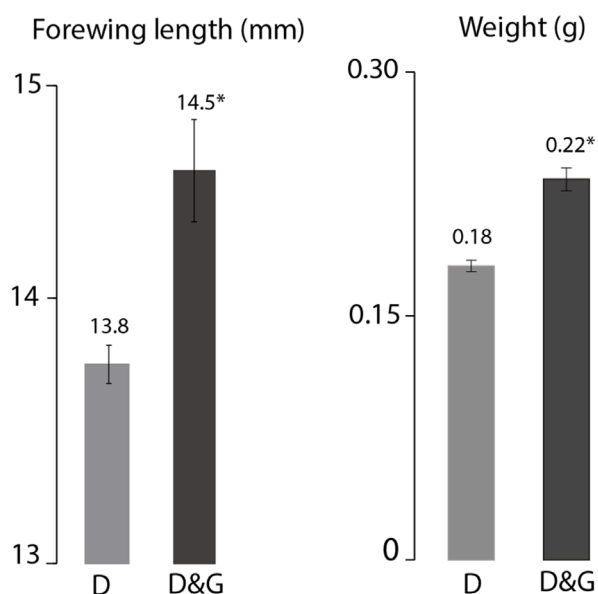


Fig. 5 Forewing length (mm) and weight (g) of drones produced in D&G or D colonies.

\*Significant differences ( $P < 0.05$ ).

Table 1

Average values for the different colony classes: drones and gynes producing colonies (*D&G*), or only drones producing colonies (*D*).

Variable	<i>D&amp;G</i>	<i>D</i>
Number of colonies	16	11
Days in captivity	99*	118
Number of workers <sup>a</sup>	118*	177
Number of drones <sup>b</sup>	78*	23
Number of gynes <sup>b</sup>	51	-
Longevity of the founder queen <sup>c</sup>	147	146
First adult drone <sup>c</sup>	160	172
First operculated cell (gyne larvae) <sup>c</sup>	148	-
First adult gyne <sup>c</sup>	172	-

<sup>a</sup> total number of workers when a colony was transferred to free foraging,

<sup>b</sup> total produced in first cycle,

<sup>c</sup> days (from time of capture of the founder queen).

\* Significant differences between the two classes of colonies ( $P < 0.05$ )

$$P_{D\&G \text{ colonies}} = \frac{e^{(2.648692 - 0.01719 w)}}{1 + e^{(2.648692 - 0.01719 w)}}; p < 0.05$$

### Resource allocation in *D&G* and *D* colonies

The analysis of covariance of cumulative pupae on cumulative cells for workers and drones for *D&G* and *D* colonies generated a curve that started higher for *D&G* than for *D* colonies, and indicated a greater colony capacity for provision food (i.e., higher colony vigor). The

slopes of the curves at  $t_0$  (i.e., the time of queen death) and  $t_{-20}$  were significantly lower for *D&G* colonies than for *D* colonies ( $P < 0.1$ ). Furthermore, in *D&G* colonies the curve for time  $t_0$  had a lower slope than the curve at  $t_{-20}$  (Fig. 8A). This result indicated that the number of pupae produced per cell was lower for *D&G* colonies at this point in colony development showing that more resources were being allocated to the production of higher-energy-cost gynes than to workers.



Fig. 6 Production of sexual forms in *D&G* colonies. A: operculated cell (Gyne larvae); B: gyne pupae; C: worker or drone pupae; D: pollen storage cell.



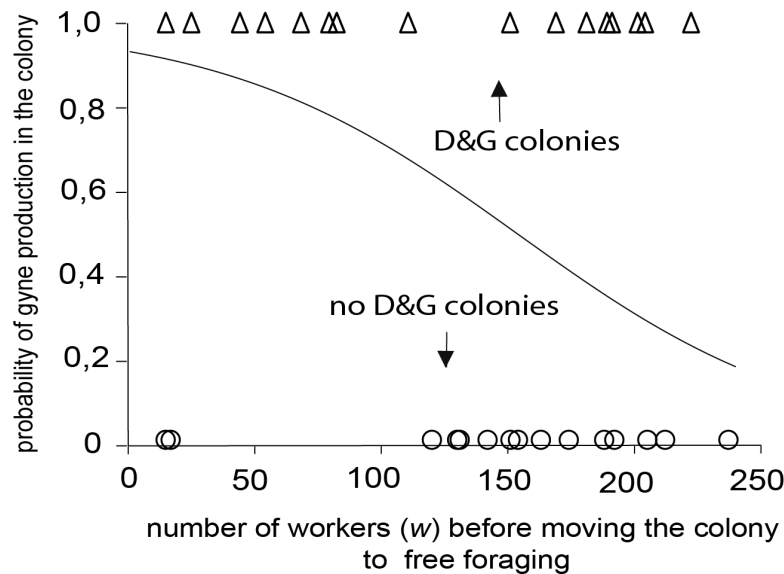


Fig. 7 Logistic model describing the probability of producing gynes at the end of the colony cycle as a function of the number of workers produced in captivity before the colonies were moved to field conditions.

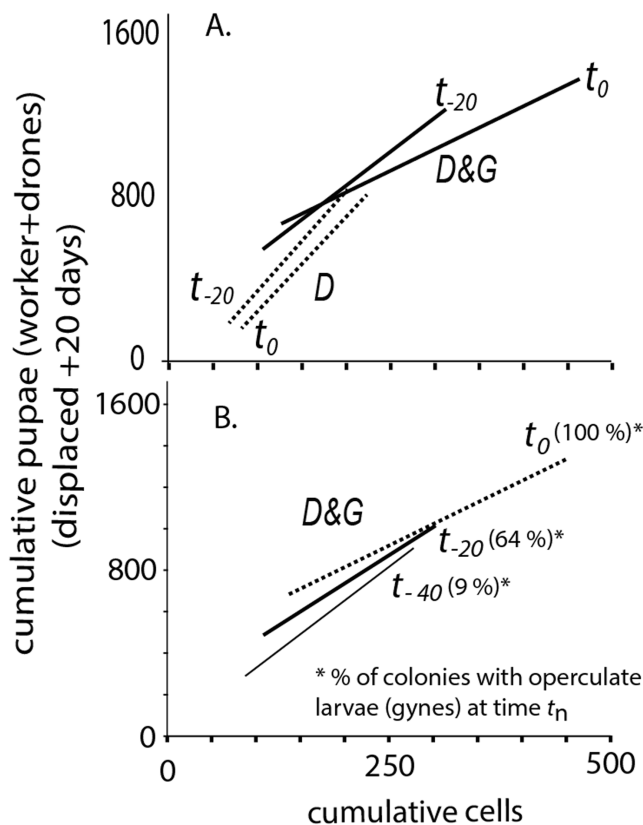


Fig. 8 Plots of cumulative pupae (workers + drones) (displaced +20 days), in 8 D&G and 11 D colonies, on the cumulative number of cells, at different times during colony development relative to the time of the death of the queen ( $t_0$ ,  $t_{-20}$ ,  $t_{-40}$ ). A: Covariance analysis using class of colony (D&G or D) as a treatment, and cells as a covariate at times  $t_{-20}$  and  $t_0$ . B: Multiple regression analysis for D&G colonies at times  $t_0$ ,  $t_{-20}$  and  $t_{-40}$  (see methods section).

A multiple regression analysis confirmed the progressive decrease in the slope of cumulative pupae on cumulative cells as D&G colonies developed to the *sexual phase* (Fig. 8B). The slopes of the curves were consistently in the direction  $t_{-40} > t_{-20} > t_0$ ; specifically, at time  $t_{-40}$  a mean of 3.26 worker pupae/cell was produced, 2.66 pupae/cell at  $t_{-20}$  and at 2.06 pupae/cell at  $t_0$ . This confirmed the hypothesis that before the death of the queen, the colonies were preparing for the *sexual phase* of colony development. This was further borne out by the fact that at  $t_{-40}$ , 9% of the colonies had operculated gyne larvae, 64% at  $t_{-20}$  and 100% at  $t_0$ . The number of pupae per cell, the number of cumulative cells, and cumulative gynes and drones were depicted for three developmental outcomes of D&G colonies characterized below as *types 1, 2 and 3 colonies* (see Fig. 9).

**Type 1 colonies** - Four colonies ( $n = 4$ ) produced sexual forms faster than the other colonies (*type 1*, Fig. 9 A-C). During the *linear phase* in these colonies, 3.7 cells were added per day (see isocline *a* in Figure 9B). The number of pupae/cell increased before stabilizing at a plateau of 3.5 pupae/cell for a period of about 34 days and then declined. The beginning of the plateau coincided roughly with the time when the first gyne-eggs were laid by the queen or about 40 days before first adult gyne emerged (see

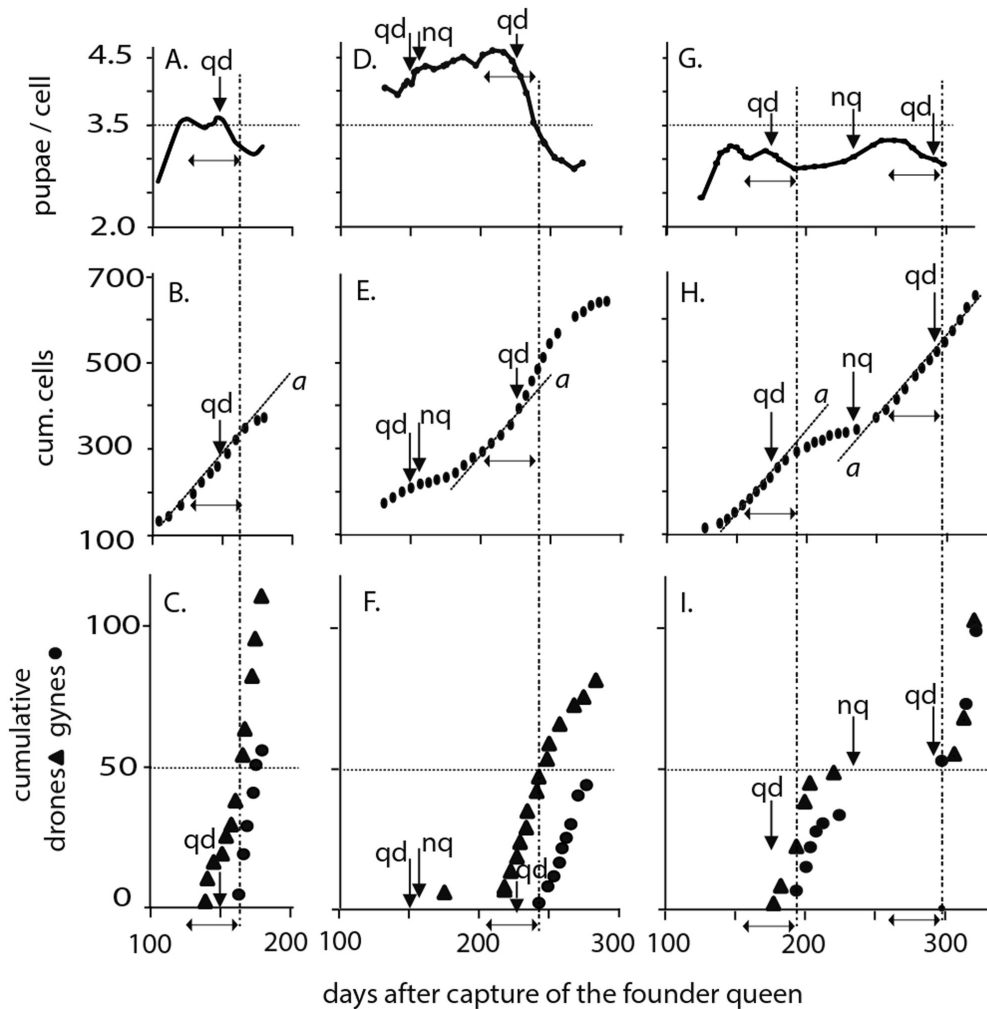


Fig. 9 Comparison between three types of colony development. Type 1 (A-C) Mean values of the colonies ( $n=4$ ) that produced sexual forms in the shorter period of time; Type 2 (D-F) Colonies that lost the founder queen and were invaded by a new queen ( $n=2$ ). Type 3 (G-I) Colony with two production cycles ( $n=1$ ). A, D, G: Cumulative number of pupae (worker + drone) per cell. B, E, H: Cumulative number of cells with the isocline  $a$  with slope=3.7 was calculated from data in B, and used as a reference value for E and H. C, F, I: Cumulative number of drones and gynes. In the figures,  $\downarrow qd$  = queen death,  $\downarrow nq$  = new queen; the vertical dashed lines = time the emergence of the first gyne; double arrow ( $\leftrightarrow$ ) indicates the estimated period from first gyne egg to first gyne adult (40 days).

the horizontal double arrow in Fig. 9) and the declined that occurred just after queen death at 147 DAC. The first adult drone appeared at 140 DAC, when the first gyne pupae were observed and the first adult gyne emerged on 161 DAC. An average of 121 drones and 60 gynes were produced in the *D&G* colonies (2:1 ratio). The average duration of the colony cycle from the initial capture of the queen to the production of the last gyne was 193 days. The values for *type 1* colonies were used as references for comparisons to *types 2* and *3* colony development.

*Type 2* colonies - Two colonies ( $n = 2$ ) were

classified as *type 2* and were characterized by very slow initial colony growth even though the longevity of the founder queens was 150 days and similar to the longevity of *type 1* queens (Fig. 9 D-F). After the death of the queen, the *type 2* colonies were reactivated by new queens from the outside that initiated a *linear phase* of colony growth that was steeper than in *type 1* colonies (Fig. 9B vs. 9E). Compared to *type 1* colonies, the first gyne produced by the new queens emerged earlier (85 days vs. 161 days). By the time *type 2* colonies were transferred to the field, they were bigger than *type 1* colonies; consequently, the ratio pupae/cell was higher

for *type 2* than for *type 1* (4.0 vs 2.5 pupae/cell) (Fig. 9A vs. 9D). The ratio of pupae/cell increased and plateaued at 4.4 for *type 2* vs. 3.5 for *type 1* (Fig. 9A vs. 9D). Gynes started to emerge 15 days after new queen death in *type 2*, which was the same as in *type 1* colonies. Compared to *type 1* colonies, *type 2* colonies produced fewer drones (79) and gynes (43) but the ratio was the same (~2:1). A few drones emerged after the death of the founder queen *type 2* colonies.

*Type 3* colonies - The third group of colonies (n=3) successfully produced second or even third cycles of drones and gyne, but each cycle was produced by a new queen. All three colonies exhibited this behavior, but only one is illustrated in Fig. 9 G-I. The number of cells per day during the *linear phase* was very similar in the two cycles, and were the same as in *type*

*1* colonies (Fig. 9B vs. 9H). The ratio of pupae to cells grew to a plateau of 3.0. The longevity of the founder queen was 179 days and the first gyne emerged 3 days later after its death. Compared to *type 1* or *type 2* colonies, fewer sexual forms were produced in the first cycle (47 drones and 32 gynes). Fourteen days after the emergence of all first-cycle gynes, a new queen took control of the colony and the ratio of pupae/cell increased to 3.3. In the second cycle, the time to first drones and gynes was reduced from 182 to 63 days, but the longevity of the new queen was only 57 days. The first gyne of the second cycle emerged six days after the death of the queen. 48 drones and 45 gynes were produced in the second cycle for a ~1:1 ratio. The ratio drones to gynes over the two cycles was 1.23:1, which is lower than in single-cycle *type 1* colonies. Using the number of sexual forms produced per queen as a metric of colony vigor, the colonies were classified as *type 1* (vigor = 1) > *type 2* (=0.68) > *type 3* second cycle (=0.57) > *type 3* first cycle (=0.46).

### Temperature variation inside the colonies

Internal temperatures were recorded for six differently sized colonies in the field and compared to ambient temperatures. The size of the colony was based on the number of workers present at the time the colonies were transferred to the field. The colonies were classified as small (15±5 workers), medium (45±10) or large (170±10).

The maximum temperatures inside the colonies were higher than ambient for all colonies, with maximum and average temperatures being higher in large and medium size colonies than in small colonies ( $P<0.05$ ) (Fig. 10). Minimum temperatures were significantly higher than ambient in large and medium size colonies, but not in small colonies.

Hourly temperatures were compared to ambient temperatures throughout all phases of colony development in a medium size colony with 44 workers (Fig. 11). Mean internal temperatures in the colony were 20.8°C during the *linear phase*, 27.4°C during the *sexual phase* and 18.7°C during the *post-sexual phase*. These average internal temperatures were higher than ambient during

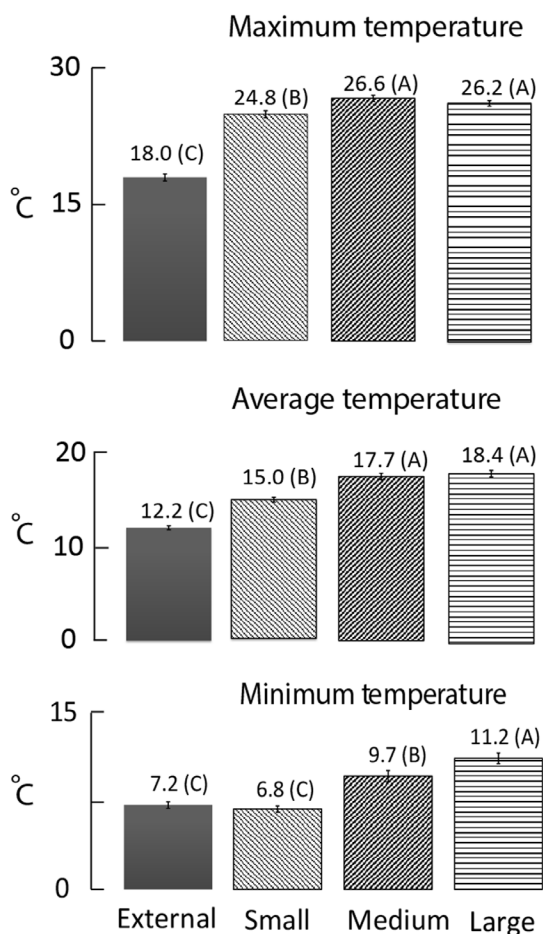


Fig. 10 Comparison between the internal and external temperatures of colonies with different number of workers when transferred to the field (see text). Bars with different letters show significant differences using Tukey's test).

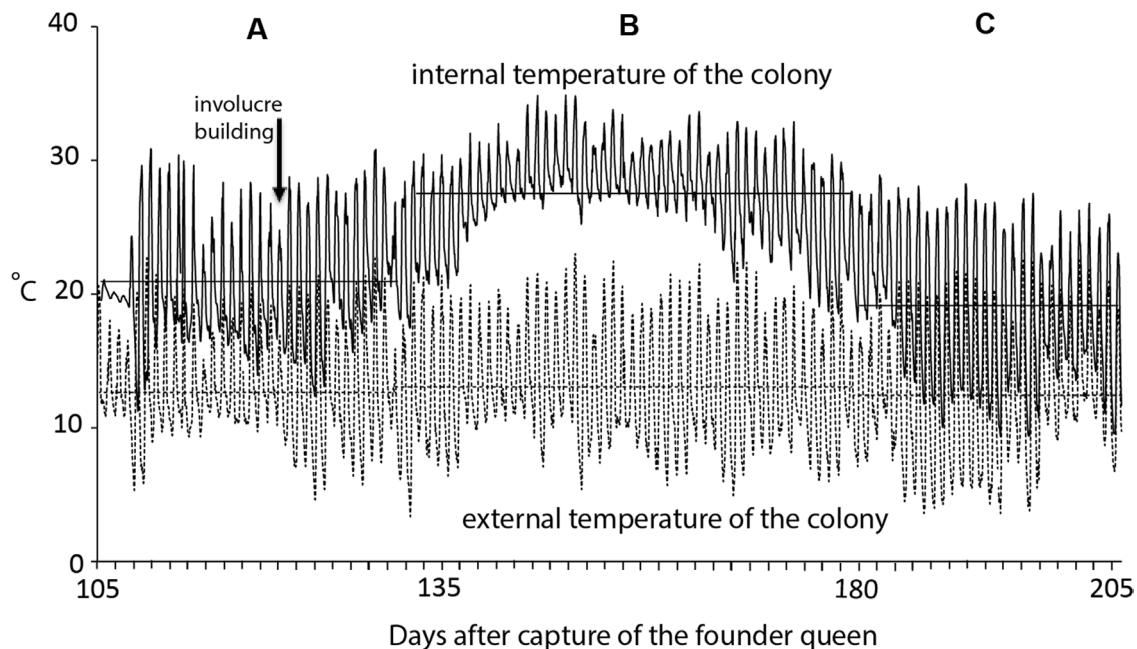


Fig. 11 Comparison between of hourly internal (upper line, \_\_\_\_\_) and external temperatures (lower line, ----) during three phases of D&G colony development with an initial 44 workers when transferred to the field. The horizontal lines are the average temperatures during the different periods of colony development: (A) the linear phase of colony development, (B) the sexual phase and (C) the post-sexual phase. The time of involucre building is indicated by the down arrow.

all phases of colony development: 7.9°C higher during the *linear phase* (Fig. 11A), 14.3°C during the *sexual phase* (Fig. 11B), and 6.1°C during the *post-sexual phase* after the emergence of the sexual forms (Fig. 11C). The construction of the waxy involucre by the workers during the linear phase starting at 122 DAC (vertical arrow in Fig. 11) served to decrease heat loss in the colony.

## DISCUSSION

### Cell building and oviposition

When the first workers emerged, the colony began to transition from the *initiation phase* to the *linear phase* of growth. The slope of the *linear phase* (i.e., cells/day) was a measure of queen vigor. Another measure was the number of worker adults reared from the cells. The average number of cells built per day by a queen of a *B. atratus* colony studied by Sakagami, Akahira & Zucchi (1967) in Brazil was  $2.1 \pm 0.3$  with an average of  $8 \pm 2$  eggs per cell, all of which survived to adults. In Colombia, González, Mejía & Rasmussen (2004) observed that 1.5 cells were built per day, but 52% of the cells were destroyed by the workers, and the same

rate was reported for *B. terrestris* by Duchateau & Velthuis (1988). In our field colonies with the highest rates of cell production during the linear phase of growth (*type 1* colonies), a mean of 3.7 cells was produced per day with the mean number of pupae/cell increasing from 2.5 to a plateau of 3.5. Some cell had as many as 8 pupae. The plateau of pupae/cell was reached 20 days after the linear increase in cell production began. We postulate that the transition to a near constant number of pupae per cell marks the initiation of the *sexual phase* of colony development, and reflects the higher investment costs of rearing immature gyne compared to rearing immature workers. The number of cells/day and pupae/cell in *type 1* colonies (see text) is a metric for the maximum growth potential of *B. atratus* colonies.

### Production of sexual forms

In nature, maximizing the production of sexual forms, especially gynes, is the objective of *B. atratus* colonies. Prior studies by us have shown that captive fed *B. atratus* colonies unable to forage in the field rarely reach the *sexual phase*. Furthermore, our data shows that early transfer



of colonies from the laboratory to the field increases the probability of *B. atratus* producing gynes, even though the colonies may have few workers at the time of transfer. On average, colonies developing into *D&G* colonies were transferred to open conditions 19 days earlier than colonies producing only drones. We hypothesize that essential nutritional factors are lacking in the diet of colonies reared in captivity. The behavior of handling and processing of pollen by the workers and presenting it to the immature siblings is disrupted, and the overabundance of food fails to stimulate initiation of sexual form production in the colony.

In contrast, workers that forage in the field choose what food to gather and they participate in all phases of larval rearing and feeding. Bumblebees are known to prefer high quality pollen with more total protein and higher essential amino acid content (Leonhardt & Blüthgen, 2012). For example, red clover (*T. pratense*) is a very important resource of nectar and produces high quality pollen for many species of bumblebees (*B. lucorum*, *B. hortorum*, *B. pascuorum*, *B. hortulanus*, *B. funebris*), especially in urban areas (Brian, 1951; Hanley et al., 2008; Hennig & Ghazoul, 2011; Rubio, 2012). *T. pratense* was an important food resource for *B. atratus* in our study area with continuous flowering occurring throughout the year that peaked during December to February (Lobatón Cure & Almanza, 2012). The maximum number of sexual forms in our study occurred in February in colonies transferred to the field the prior December (see Figure 3). We posit that success in producing gynes in these colonies was linked to the availability of an abundant supply of *T. pratense* inflorescences.

### Colony development

In *B. terrestris*, some colonies exhibit an early *switching* to the production of sexual forms at the time the first male egg are produced by the queen. Some *B. terrestris* colonies have late *switching* and produce few or no drones, but some may produce many gynes (Duchateau & Velthuis, 1988; Duchateau, Velthuis & Boomsma, 2004). In *B. perplexus*, the first queens emerge

3.2 days after the first drones (Pomeroy & Plowright, 1982), and in *B. melaleucus*, colonies start producing gynes earlier than drones (Hoffmann, Torres & Neumann, 2004). Cnaani, Schmid-Hempel & Schmidt (2002) studied 11 colonies of *B. impatiens*, of which three failed to reach the *sexual phase*, four produced drones and gynes, two colonies produced only gynes and two produced only drones. In *B. impatiens*, 9% of the workers in old colonies had mature oocytes and were able to lay eggs, but never did so in the presence of the queen or during the *post-sexual phase* (Cnaani, Schmid-Hempel & Schmidt, 2002).

Zucchi (1973) observed in *B. atratus* colonies that 90% or more of all drones produced were developed from eggs laid by workers. In our studies, workers were not observed laying eggs in the presence of a viable queen but did so when the queen was weak or occasionally when the colony was in the *post-sexual phase*. In our study, all colonies that reached the *sexual phase* produced both drones and gynes, but no colonies produced only gynes.

In *B. terrestris*, colonies reared in chambers at 28°C and 60% RH reached the *linear phase* 27 days after colony initiation, and 20 days later the queens laid the first drone egg (Duchateau & Velthuis, 1988). In *B. atratus*, the duration of the *initiation phase* was more variable. Limiting conditions such as temperature, resources shortfall and poor queen vigor could extend the *initiation phase* and decrease the number of cells produced per day during the *linear phase* of colony development. The *linear phase* in high vigor *type 1* colonies started at 100 DAC, which was much earlier than in *type 2* or *type 3* colonies. In *type 1* the first drone egg was laid 10 days after the initiation of the linear phase.

The emergence of drones occurred on average thirteen days after the queen death in *D&G* colonies and 26 days after the queen death for the *D* colonies. We suggest that drones from *D&G* colonies were produced by the queen while those from the *D* colonies were from eggs laid by the workers after the queen lost the control of the colony. In addition, in our studies, drones from *D&G* colonies were larger than drones from

*D* colonies. This has an implication on fitness as some studies suggest that larger drones have more sperm and transfer a higher quantity of sperm during copulation (Garófalo, Zucchi & Muccillo, 1986; Baer, 2003). Our results differ from Silva-Matos & Garófalo (1995, 2000) who found no differences in drone size between queen-led and queen-less *B. atratus* colonies.

Sexual production in bumblebees is strongly drone biased (Owen, Rodd & Plowright 1980; Owen & Plowright, 1982; Fisher 1987, 1992; Röseler & van Honk, 1990; Beekman & Van Stratum, 1998). In an analysis of eleven populations of seven *Bombus* species, Bourke (1997) calculated a male-biased ratio of 3:1. Using linear regression with a zero intercept throughout all *B. atratus* *D&G* colonies, we found that the number of gynes to total gynes plus drones gave a drone biased ratio of ~2.27:1 (slope = 0.44). Nevertheless, there is an ample variation among individual colonies, which show a comparatively high number of gynes (87 of 136 total gynes plus drones) to a low number of gynes (41 of 370 total gynes plus drones).

### Reactivation of colonies

An important aspect of biology of *B. atratus* is its capacity to reactivate colonies and produce successive generations (Sakagami, Akahira, & Zucchi, 1967; Zucchi, 1973; Silva-Matos & Garófalo, 1995; Cameron & Jost, 1998; Riaño et al. 2014). Colony reactivation occurs when a new queen takes control of the colony after the founder queen dies or loses vigor. The entrance of a new queen into a colony and co-existence with the old queen for short periods of time has been observed in *B. atratus*. Riaño et al. (2014) described a sequence of three invasions by new queens to the same nest as follows: the old queen was killed by the invading queen that then controlled the nest for a short period of time, but that in turn was replaced by a new invading second queen that successfully produced sexual forms. Silva-Matos & Garófalo (1995) reported successive cycles in a colony controlled by sibling queens that occasionally remained in the nest and successfully defend territory. In four of our *B. atratus* colonies, two

queens coexisted from 18-39 days, but only one developed the colony to the *sexual phase*. The coexistence of a young and old queen is not necessary for reactivation of a *B. atratus* colony, as a new queen can reactivate an orphan colony and successfully produce a generation of sexual forms.

In our studies, the mean duration of a complete cycle from the capture of the queen to the emergence of the last gyne was 193 days. This is equivalent to 2374 degree days above 10°C at a mean nest temperature of 22.3°C. The mean time for completion of a cycle of a *B. atratus* in Brazil was  $170 \pm 15$  days. When a new queen reactivates an orphan colony, the time required to produce sexual forms is half the time of the first cycle. In a perennial *B. atratus* colony studied by Zucchi (1973) in Ribeirão Preto, Brazil, two to three gyne production cycles per year were obtained with a mean of 128 gynes per cycle. The colonies had access to the open field and were able to produce three cycles because they were kept inside a chamber with temperature controls and bypassed the *solitary phase* of colony foundation during the second and third cycles. In our studies, a mean of 51 gynes was produced in the first cycle with a decline to 44 in the second cycle. The mean longevity of the founder queens in our studies was similar in both *D&G* and *D* colonies (146 days, 1796 degree days), while in Brazil the mean longevity was  $114 \pm 41$  days (Zucchi, 1973).

### Temperature inside colonies

Bumblebees regulate the temperature of the nest for the developing brood (Heinrich, 2004) using the metabolic heat of the workers and the insulating effects of the wax involucre lining the rearing chamber. Regulating the nest temperature several degrees above the external temperature shortens the developmental time of the brood and is an important consideration in some areas where the time available for amassing resources prior to producing sexual forms may be short (Morse, 1982). We note that ventilation of the nest can also be used to decrease unfavorable high nest temperatures. Considerable energy savings are possible when

the founding queen finds a place for the nest to take advantage of existing insulation that helps retain metabolically heat (Heinrich, 2004). *B. atratus* colonies with more than 50 workers were able to maintain an average temperature of 18°C (6°C above the ambient temperature) and a minimal of 10.5°C (3.5°C above the ambient temperature). However, smaller colonies with fewer workers were unable to fully regulate nest temperature during early colony development. By way of comparison with other *Bombus* species, nest temperatures for *B. pascuorum* were reported to vary between 24–33°C (Brian, 1952; Barrow & Pickard, 1985) and 20–27°C for *B. polaris* (Richards, 1973).

The average internal temperature of the *B. atratus* colonies changed during the three phases of colony development (e.g., 20.8°C, 27.4°C and 18.7°C respectively). Hasselrot (1960) and Wojtowski (1963) also reported differences in temperature in bumblebee nests during the different phases, where the initial and terminal phases had low fluctuating temperatures. In our study, the middle phase had constant temperatures independent of external meteorological conditions. This is the period when the largest numbers of workers are present and when drones and gynes are being produced. Temperatures during this middle period reported for *B. agrorum*, *B. ruderarius*, *B. lapidarius* and *B. silvarum* were 32°C and 33°C for *B. terrestris* (Hasselrot, 1960; Wojtowski, 1963).

### Concluding remarks

An important bionomic characteristic of bumblebee species is that they are either pocket makers or pollen storers (Sladen, 1912; Sakagami, 1976). All commercially reared bumblebee species are pollen storers, as pocket makers have generally proven less suitable for domestication because they usually accept only the pollen that is put inside the pockets by the beekeeper, and this behavior is expensive and time consuming to accommodate (Velthuis & Doorn, 2006). The best rearing results with pocket makers leading to the production of gynes were obtained when colonies were allowed to forage for their own food soon after

the emergence of the first workers (Griffin et al., 1991). *B. atratus* is a pocket maker species, and in our studies early transfer of the colony to the field was an important factor in successful colony development leading to the production of gynes.

A colony is successful when it produces new gynes, but this depends heavily on the vigor of the founding queen, the quality and quantity of food available during colony development, nest placement and weather. A good combination of these factors minimizes the duration of the *initiation* and *linear phases* of colony growth and increases the production of the sexual forms. The vigor of the queen is a difficult variable to assess, but our results show that under field conditions, a vigorous colony can build a maximum of 3.7 cells per day and rear 3.5 adult workers per cell during the *linear phase* of colony growth. In our study, such colonies produced an average of 60 gynes and 121 drones per cycle. According to Michener (2007), the apparent uniformity of bumblebee morphological characters are monotonous when compared to the Euglossini and especially the Meliponini. However, bumblebees can exhibit great behavioral plasticity in colony development. For example, colonies that are delayed in building a worker population can be reactivated by a new queen and still be capable of producing gynes. The adaptive reactivation of colonies enables *B. atratus* colonies to achieve up to three cycles per year in the face of environmental and other hazards. Several colony survival strategies were observed in *B. atratus* colonies by Garófalo (1974) and Sakagami (1976): 1) Monogynic colonies with production of gynes that leave the nest and individually initiate a new colony; 2) Monogynic colonies with sibling queens ovipositing and defending territories in the same nest (polygyny) until just one queen remains; 3) Orphaned or weak colonies where large workers mate and defend territories until a new queen from outside takes control of the colony; 4) Colonies in *post-sexual phase* where large mated workers swarm and are joined by workers to form other nests to initiate a colony which survives until a queen enters and takes

control. We observed only strategies 1) and 3) in our study.

The observed plasticity in *B. atratus* colony development and the limitations on the number of colonies that can be observed make it difficult to interpret and to compare the different growth patterns of the 31 colonies in our studies. Among the important factors influencing development of *B. atratus* colonies were the vigor of the founding queens, the influence of time in captivity before the colonies were put in the field, and the kinds and levels of resource available to them. Integrating the disparate observations and data on *B. atratus* in a dynamic manner requires the development of a model that captures the time varying rates of resource acquisition and allocation of each colony that includes the behavioral and physiological responses to variable weather and resource availability. A physiologically based demographic model (see Gutierrez, 1992) has been used to model the processes of resource acquisition and allocation in numerous plant and animal species, and similar applications are apparent for modeling *B. atratus* colony development. Based on results obtained in this and other studies here discussed, the behavioral plasticity observed in *Bombus atratus* is a good starting point to explore new possibilities for rearing and optimizing the production of this species to be used in crop pollination.

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